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Fear Conditioning Effects on Sensitivity to Drug Reward

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14. ABSTRACT

The overall hypothesis is that mice undergoing repeated exposure to an aversive stimulus that is paired with neutral cues would develop ongoing fear responses (freezing behavior) to these cues, enhanced approach responses to drug associated contexts, and neural plasticity in relevant brain regions. Mice demonstrated freezing behaviors over 80% of the time when exposed to the context and tone cues (vs. 10% freezing behavior in the control group). In addition, fear extinction trials (20/day) employing repeated exposure to the context and tone cues without footshock administration abolished freezing behavior in previously fear-conditioned mice. Moreover, fosB levels were elevated by more than a third in the prelimbic and infralimbic cortices of fear extinguished mice. After place conditioning, both sham and fear conditioned mice exhibited a robust preference for the morphine associated side. Thus, fear extinction produces activation of neural plasticity factors in pre- and infralimbic cortices but acquisition of conditioned fear did not alter subsequent acquisition of conditioned opiate reward. This innovative approach will identify neural mechanisms and treatment approaches for post-traumatic stress disorder and addiction.

15. SUBJECT TERMS

Fear conditioning, place conditioning, morphine, extinction, fosB, cortex, mouse

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Introduction: Subject, Purpose, and Scope of the Research

This novel translational proposal examines how repeated stressor exposure in an animal model produces learned fear responses which emulate post-traumatic stress disorder (PTSD) and how such fear responses enhance sensitivity to drug reward and addiction-related behaviors. In the Veteran population, PTSD and substance use disorders are frequently comorbid conditions. An estimated 20% of returning Operation Iraqi Freedom (OIF) Veterans have developed PTSD; substance abuse is also prevalent with 35% of OIF Veterans exhibiting alcohol abuse (Hoge et al. 2004). These comorbid disorders are often chronic and disabling, their etiologies and neural mechanisms are unknown and only partially effective treatments are available (Rauch et al., 2006). This study will link PTSD-associated behaviors to subsequent addiction and will emulate aspects of the experience of traumatized Veterans. This innovative approach will identify convergent neural mechanisms and concomitant treatment approaches for PTSD and addiction.

Body: Research Accomplishments from the Statement of Work

Four tasks outlined in the approved Statement of Work and listed below together with the record of relevant research findings. Statistical tests of significance have been performed and exhibited within the embedded figures/table. Both positive and negative findings are included together with recommended future work to better address the research topic.

The ***first step*** is to implement the fear conditioning (vs. control manipulation) procedure in mice to produce freezing behaviors and measure their time course of extinction. Brains will be collected after testing to measure the activation of c-Fos in limbic brain areas by using quantitative IHC.

First Step objectives involved validation of fear conditioning acquisition/extinction procedure followed by assessment of regionally specific neuroadaptations which accompany these forms of learning. The experimental timeline, shown in the figure below, exhibits the order and details of experimental manipulations and dependent measure collection. Mice were first fear conditioning on a single day, and then either exposed to extinction training or a delay in the absence of re-exposure to the fear conditioned cues/context (sham extinction) over the subsequent four days. Following the expression of fear conditioning/extinction, brains were harvested for quantitation of neuroadaptations accompanying learning.

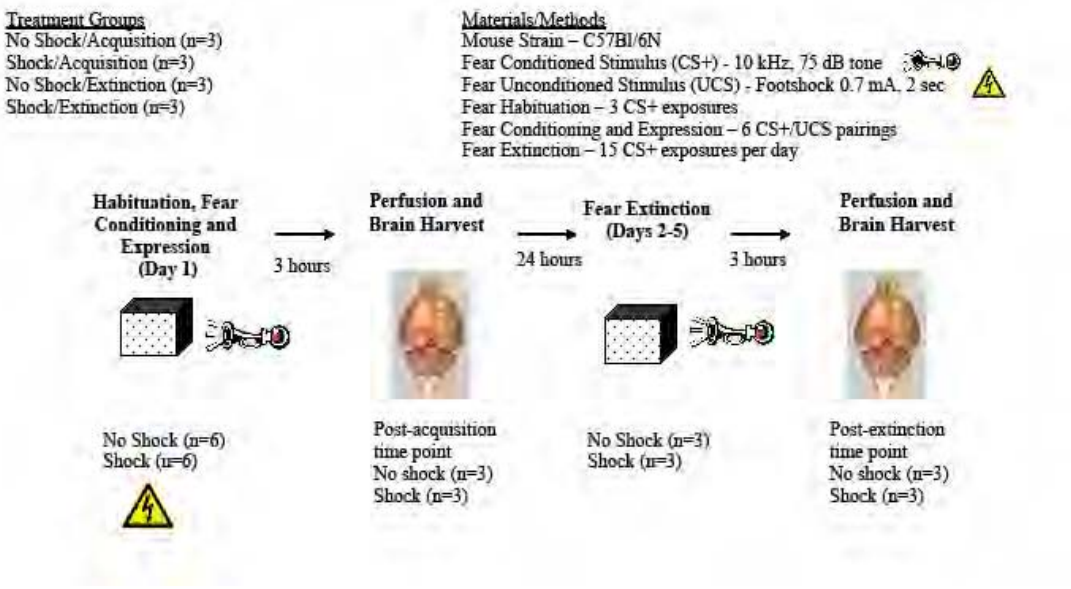


Figure Legend -The sequence of experimental procedures necessary to condition and extinguish freezing behavior shown from left to right in the schematic timeline above. Brains were harvested from a subset of conditioned and extinguished mice.

Results of the behavioral component of the fear acquisition/extinction study revealed that exposure to the tone/context cues in the presence of a mild footshock elicited absence of movement (freezing) in conditioned mice, but not in controls which were not exposure to the footshock stimulus. This acquisition of fear conditioning result is exhibited in the figure below.

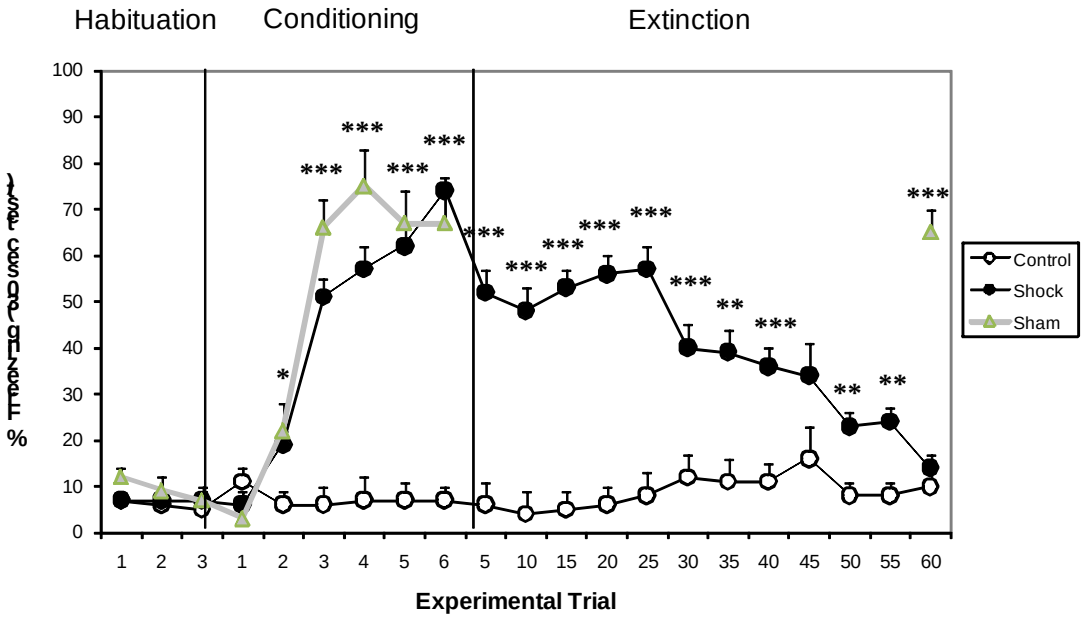


Figure Legend – Acquisition and extinction of conditioned fear. The figure exhibits percentage of time spent freezing (mean ± SEM) in a distinctive testing compartment with auditory cues by mice during habituation, conditioning and extinction trials. On the first experimental day, mice were familiarized with the compartment and tone (10 kHz, 75 dB) over three trials (habituation) and then exposed to tone alone (control group, n=11) or a fear conditioning stimulus (footshock, 0.7 mA, 2 sec, n=11) co-terminating with the tone. During extinction trials, mice were exposed to the conditioned context/tone for 60 trials over four days (15 extinction trials/day) in the absence of shock administration. A sham extinction group (n=4) was fear conditioned and tested for fear retention four days later without any intervening extinction training. All trials were conducted using a variable interval 3 minute schedule and concluded with a 30 second sounding of the tone cue during which freezing behaviors were quantified. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ relative to control

Continuing the interpretation of results presented in the above figure, mice which had been fear conditioned continued to express conditioned freezing after a four day delay (sham extinction) during which mice were handled and tested in a locomotor context, but not re-exposed to the tone/context paired previously with footshock. In contrast, extinction training resulted in a progressive weakening of the duration of conditioned freezing concluding in the abolition of conditioned fear after 60 extinction trials when the duration of conditioned freezing in the fear conditioned mice was restored to the unconditioned, control baseline.

In order to assess the persistence of the decline in conditioned freezing produced by exposure to extinction learning procedures, a spontaneous recovery procedure was performed in order to quantify rebound of fear conditioning following a post-extinction delay. The results, shown in the figure below, indicted that a one week period of time spent in the home cage following completion of 45 trials of extinction training resulted in a significant rebound of conditioned fear relative to unconditioned control mice. This finding suggests that extinction learning masks, rather than erases, original acquisition learning. This result also increases the potential utility of pharmacological adjuncts to exposure therapy procedures in order to increase the persistence of original extinction learning.

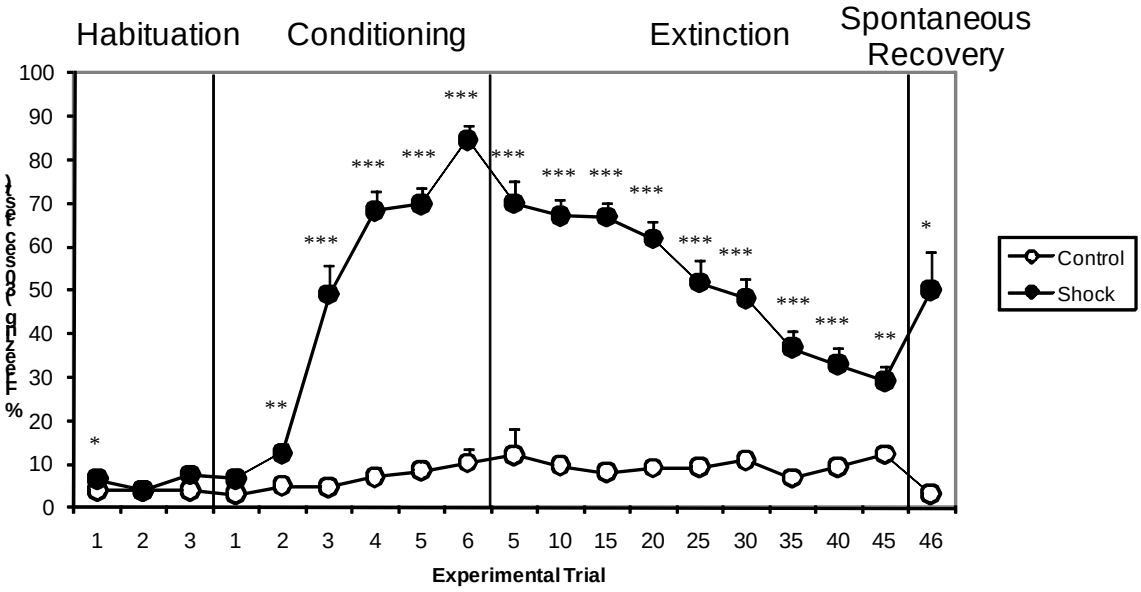


Figure Legend – Acquisition, extinction, and spontaneous recovery of conditioned fear. The figure exhibits percentage of time spent freezing (mean ± SEM) in a distinctive testing compartment with auditory cues by mice during habituation, conditioning, extinction, and spontaneous recovery trials. On the first experimental day, mice were familiarized with the compartment and tone (10 kHz, 75 dB) over three trials (habituation), then exposed to a sham procedure (control group, n=12) or fear conditioning stimulus (footshock, 0.7 mA, 2 sec, n=12) co-terminating with the tone. During extinction trials, mice were exposed to the conditioned context/tone for 45 trials over three days in the absence of shock administration. A single spontaneous recovery trial was conducted one week following the conclusion of extinction training by presenting the tone in the absence of shock. All trials were conducted using a variable interval 3 minute schedule and concluded with a 30 second sounding of the tone cue during which freezing behaviors were quantified. * $p<0.05$, ** $p<0.001$, *** $p<0.0001$ relative to control

The early/immediate gene protein, fosB, was assessed in brains harvested following the conclusion of fear acquisition and extinction conditioning (Radulovic et al., 2003). The fosB protein was quantified in a variety of specific brain regions selected due to their relevance for associative learning processes, for stress reactivity, and for encoding and retention of extinction learning. fosB protein levels were measured in a total of eight brain regions using an slicing/antibody/staining procedures and the results of these studies are exhibited in the figures and table below.

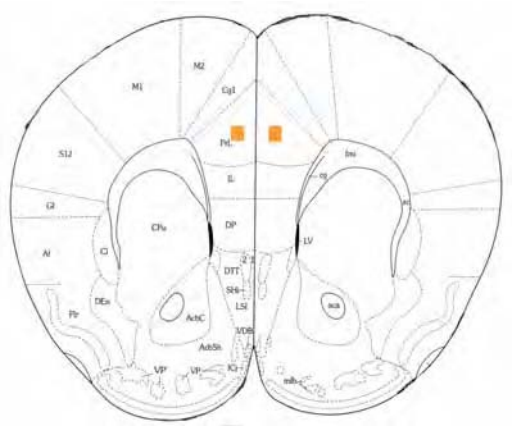


Figure Legend: The panel is a cross-sectional schematic of the mouse brain at the level of prefrontal cortex; orange blocks reflect the two regions of interest.

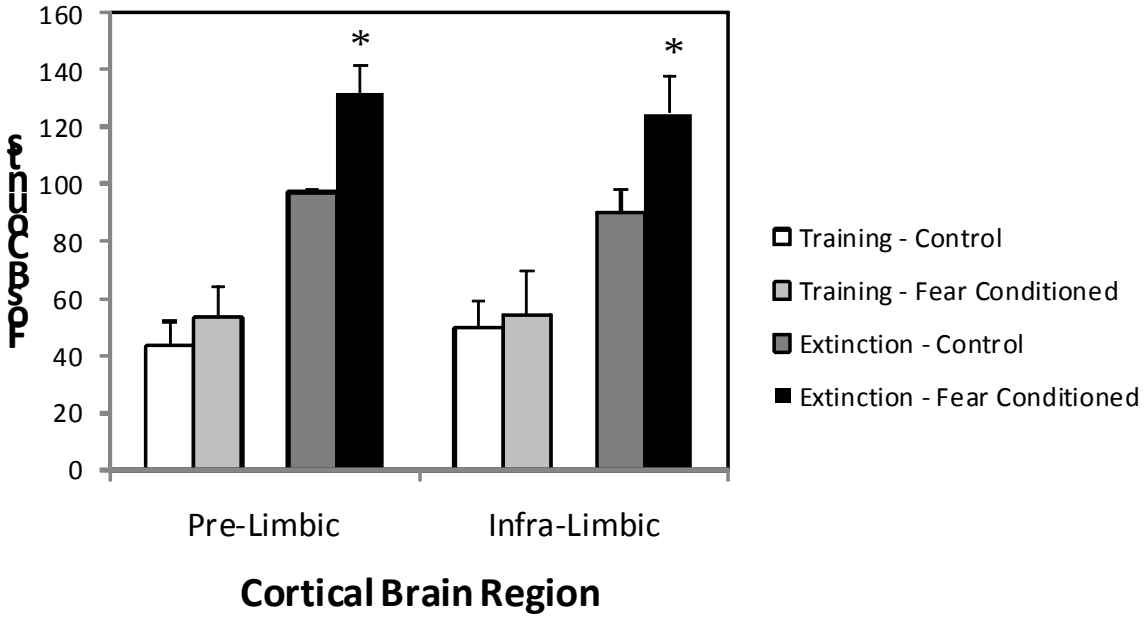


Figure Legend: FosB quantification (mean + SEM) from control and fear conditioned mice 3 hours following acquisition or extinction training in prelimbic and infra-limbic cortices. * $p<0.05$ relative to extinction controls

Results exhibited in the figure above suggest that fear extinction training, but not fear acquisition is capable of increasing expression of fosB protein within two subregions of the pre-frontal cortex, the pre-limbic and infra-limbic cortices. This pattern of prefrontal cortical activation is not surprising given the finding that this area of the brain is critical for encoding and retrival of extinction memories (Quirk & Mueller, 2008). In contrast, six additional regions of the mouse brain relevant for conditioned reward processes and emotional regulation were not impacted by the fear acquisition or extinction training procedures. As illustrated in the table below, no statistically significant differences were detected as a result of fear acquisition/ extinction relative to unconditioned control mice.

	NAc Core	NAc Shell	ACg	CPU	BLa	BLp
Training - Control	112 ± 19	70± 19	16± 4	43± 5	21± 4	13± 3
Training - Fear Conditioned	98± 21	67± 25	19± 6	36± 5	32± 15	16± 2
Extinction - Control	208± 53	128± 46	89± 5	163± 14	71± 3	59± 5
Extinction - Fear Conditioned	224± 12	167± 9	72± 16	168± 7	74± 9	62± 10

Table Legend: FosB immunoreactivity (mean ± SEM) measured in a variety of brain regions 3 hours following fear or extinction training in mice exposed to sham procedures (Control) or footshock stimuli (Conditioned). NAc – nucleus accumbens, Acg – cingulate cortex, CPU – caudate putamen, BLa – anterior basolateral amygdala, BLp – posterior basolateral amygdala

The ***second step*** is to demonstrate efficacy of the $GABA_B$ receptor agonist baclofen in blocking the development of fear conditioning and associated limbic activation. Together, these two steps constitute the initial goal of validating the behavioral, neurobiological and pharmacological aspects of conditioned fearfulness for subsequent use in opiate place conditioning studies.

Progress on the second step is pending.

The ***third step*** is to a) examine drug reward sensitivity in fear conditioned vs. control mice and b) quantify the neuronal activity and pathways using c-Fos IHC. This step is critical to test the main hypothesis that fear conditioning results in neuroadaptations in extended amygdalar-accumbal regions that results in enhanced drug reward sensitivity.

Third Step objectives involved validation of fear/place conditioning procedures. The experimental timeline, shown in the figure below, exhibits the order and details of experimental manipulations and dependent measure collection. Mice were first fear conditioning on a single day, and then either exposed to place conditioned procedures with saline or morphine over the subsequent ten days.

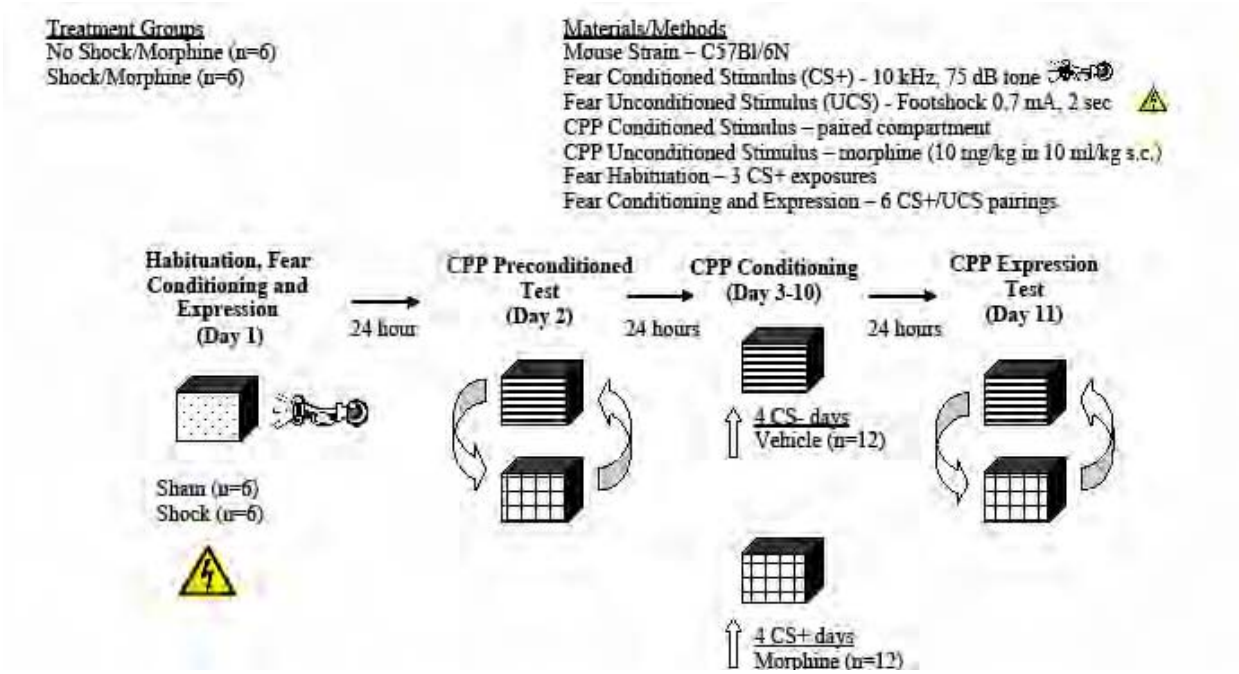


Figure Legend. The sequence of experimental procedures necessary to condition fear as well as a morphine place preference are shown from left to right in the schematic timeline above. ↑ = injection

The results indicate morphine administration within CS+ compartments of the place conditioning apparatus induced a significant morphine place preference. However, this conditioned preference was exhibited in both control and fear conditioned mice to an equivalent degree. These results, shown in the figure below, suggest that prior fear conditioning procedures do not impact subsequent morphine place conditioning. Because of these negative results, post-mortem fosB quantification studies were not performed. The outcome of this experiment could potential be improved by presentation of fear conditioned tone cues within the place conditioning environment during expression of the morphine place preference.

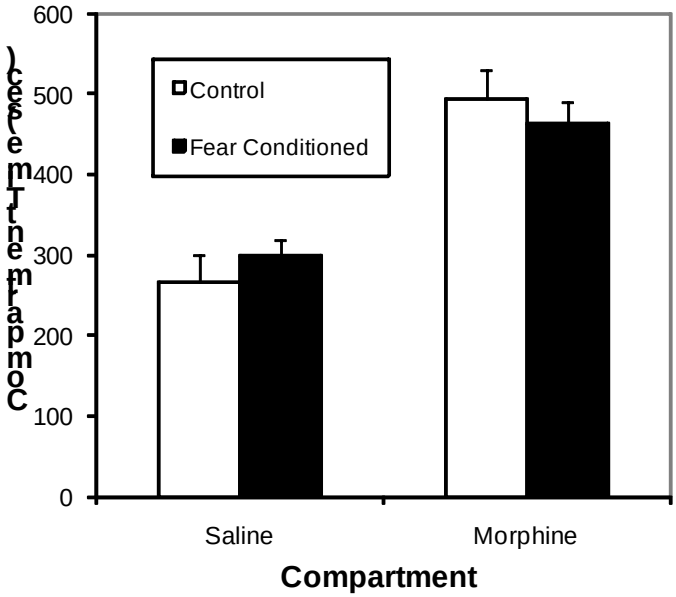


Figure Legend: Time spent in saline (CS-) versus morphine (CS+) paired compartments of the place conditioning apparatus was not altered by prior fear conditioning relative to saline conditioned controls.

The ***fourth step*** assesses the impact of baclofen given during the development of fear conditioning and measures its impact on subsequent opiate place conditioning. Each of these steps is composed of an *in vivo* behavioral component followed by *ex vivo* brain activation mapping in order to elucidate the mechanisms of fear/reward interactions.

Progress on the fourth step is pending.

Key Research Accomplishments: Bulleted List of Key Achievements

- Mice demonstrated freezing behaviors over 80% of the time when exposed to the context and tone cues (vs. 10% freezing behavior in the control group).
- Fear extinction trials (20/day) via repeated exposure to context and cue without footshock returned freezing behaviors to baseline levels.
- Fear conditioning could be partially restored following extinction training through the process of spontaneous recovery.
- FosB levels were elevated by more than a third in the prelimbic and infralimbic cortices, but not in six other brain areas of interest, of fear extinguished mice.
- After place conditioning, both sham and fear conditioned mice showed a robust and equivalent preference for the morphine associated side.

Reportable Outcomes: Manuscripts, Abstracts, Presentations, Funding, and Training

- **Manuscript** – one review manuscript related to the fear conditioning data generated during the project period has been submitted for publication: Gary B. Kaplan et al., Treatment of Addiction and Anxiety Using Extinction Approaches: Neural Mechanisms and their Treatment Implications, submitted.

- **Presentations** – three presentations (one poster and two talks) related to the fear conditioning data were generated during the project period. The three presentations are provided in the Appendix section of this report.

- **Abstracts** – two abstracts related to the fear conditioning data generated during the project period have been submitted: 1) Gary B. Kaplan et al., Prior Fear Conditioning Alters Opiate Place Preference in Mice, College on Problems of Drug Dependence,

2009; 2) Gary B. Kaplan, Fear Conditioning Effects on Sensitivity to Drug Reward, CDMRP meeting, 2009.

- **Funding** – two grant proposals for funding have been submitted based upon the results generated during the project period: 1) Neurotrophic Mechanisms in Treatments for TBI and PTSD, RR&D Scientific Merit Award, Department of Veterans Affairs, Gary B. Kaplan, P.I., 2) Modeling Comorbid Traumatic Brain Injury and Post-Traumatic Stress Disorder, Deployment Related Medical Hypothesis Development, Department of the Army, Gary B. Kaplan, Co-P.I.

- **Training** – one Boston University undergraduate has completed thesis studies in the process of collecting data during the project period

Conclusion: Summary and Significance

The most important finding of the present project period is the behavioral and neurochemical validation of fear acquisition and extinction procedures to produce conditioned freezing as well as regionally selective activation of fosB in cortical brain regions. These results confirm and extend published literature in this field and provide a high degree of confidence in the reliability and validity of the animal model data. However, additional refinement is required in order to produce behavioral carryover from the fear conditioning context into the place conditioning context and refinements of experimental apparatus and procedure are envisioned to accomplish this goal. In particular, the fear-conditioned tone cue will be administered during expression of morphine conditioned place preference as an explicit reminder of prior exposure to the aversive stimulus. The anticipated outcomes of the present studies remain the same, namely to provide a platform for detection of specific neuroadaptions or pharmacological interventions which are capable to revealing the brain mechanisms by which aversive conditioned stimuli impact subsequent drug seeking responses.

References: List of Publications Pertinent to this Report

Hoge, C.W., Castro, C.A., Messer, S.C., McGurk, D., Cotting, D.I., and Koffman, R.L. (2004). Combat duty in Iraq and Afghanistan, mental health problems, and barriers to care. New England Journal of Medicine 351:13-22.

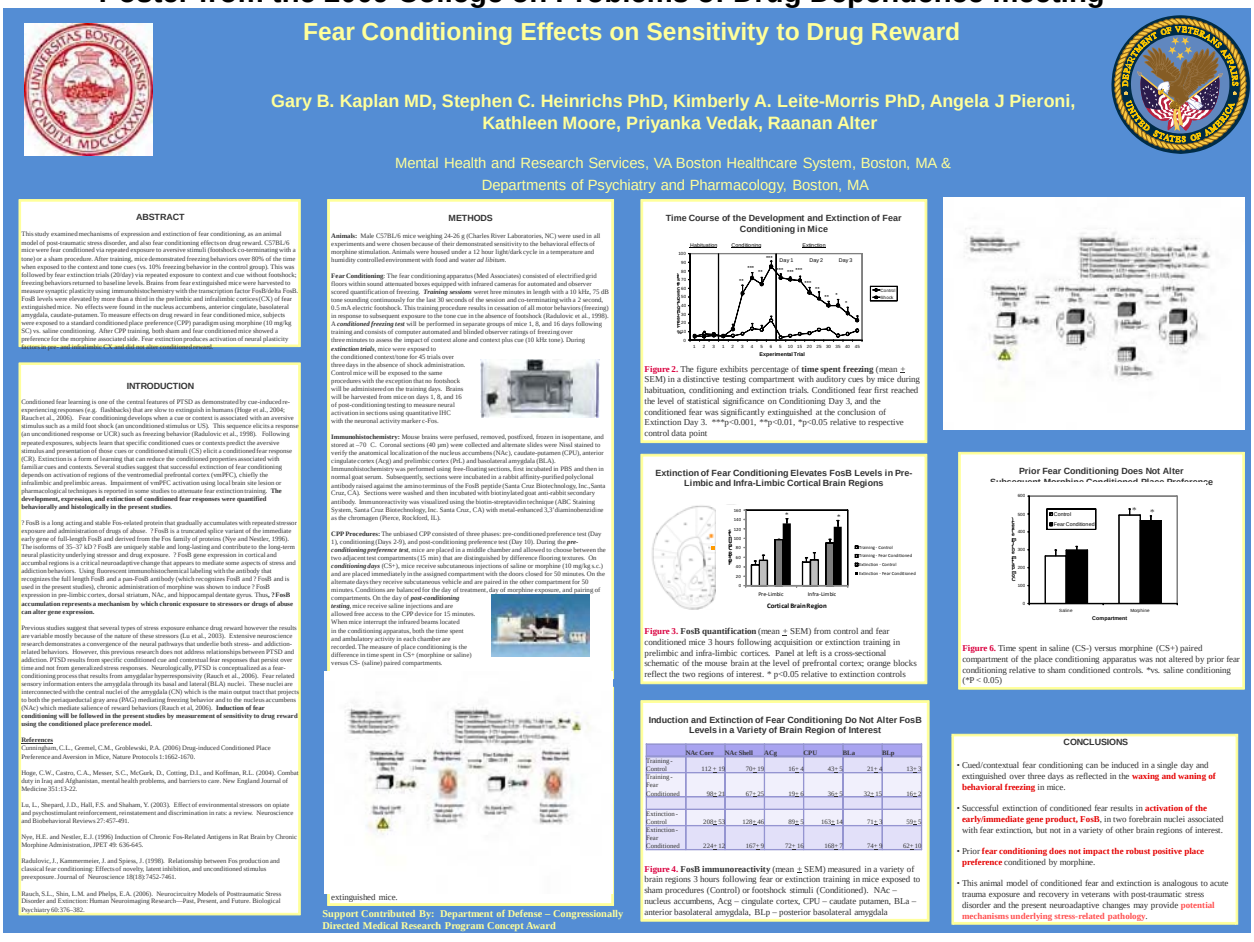
Quirk, G.J. and Mueller, D. (2008) Neural mechanisms of extinction learning and retrieval. Neuropsychopharmacology 33:56-72.

Radulovic, J., Kammermeier, J. and Spiess, J. (1998). Relationship between Fos production and classical fear conditioning: Effects of novelty, latent inhibition, and unconditioned stimulus preexposure. Journal of Neuroscience 18(18):7452-7461.

Rauch, S.L., Shin, L.M. and Phelps, E.A. (2006). Neurocircuitry Models of Posttraumatic Stress Disorder and Extinction: Human Neuroimaging Research—Past, Present, and Future. Biological Psychiatry 60:376–382.

Appendix: Supplementary Information

- **Poster from the 2009 College on Problems of Drug Dependence meeting**



- **Abstract from the 2009 College on Problems of Drug Dependence meeting**

TITLE: Prior Fear Conditioning Alters Opiate Place Preference in Mice

AUTHORS (FIRST NAME INITIAL LAST NAME): G. B. Kaplan1, 2, S. C. Heinrichs2, K. A. Leite-Morris1, 2
INSTITUTIONS (ALL): 1. Psychiatry & Pharmacology, Boston Univ Sch Med, Boston, MA, USA. 2. Research & Mental Health, VA Boston HCS, Boston, MA, USA.

ABSTRACT BODY:
Aims (Must be completed) : Conditioned fear responses are hypothesized to alter sensitivity to drug reward. A mouse model was developed to emulate post-traumatic stress disorder (PTSD) by repeated stressor exposure which produced learned fear responses.

Methods (Must be completed): Repeated aversive conditioning sessions were conducted in C57BL/6 mice using a specific environmental context and a 10 kHz, 75 dB tone cue. Fear conditioning was performed using a 2 sec 0.7 mA electric footshock in the presence of this specific context and tone. A fear retention test is performed 24 hours following training and uses automated ratings of freezing over 3 min to assess the impact of fear associated contexts and/or cues. The following day, these mice were conditioned to morphine (10 mg/kg s.c.) or saline associated compartments of using a standard conditioned place preference (CPP) paradigm and device. Post-conditioning preference was examined after 8 sessions.

Results (Must be completed): Mice acquired conditioned freezing responses after three daily pairings of this cue and context with footshock. Fear conditioned mice exhibited behavioral freezing during 40% of a three minute combined tone/context retention test on Day 4 relative to the freezing behaviors exhibited by concurrently tested control mice (3% freezing rates). On day 5, mice were exposed to CPP training followed by CPP expression testing, as measured by the difference in time spent (sec) in the CS+ vesus CS- compartments. Non-fear conditioned controls showed greater preferences to the morphine associated context (540 sec on CS+ side vs. 224 sec on CS-, $p<0.0001$) vs. Fear conditioned mice (422 sec on CS+ vs. 298 sec on CS-, n.s.).

Conclusions (Must be completed) : Fear conditioning produces freezing behavior to conditioned cues and contexts that are measureable for up to two weeks. When opiate CPP was performed during this period of conditioned fearfulness, it resulted in the attenuation of the development or expression of conditioned opiate reward. These studies suggest that PTSD development alter the conditioning of drug reward.

Support (Must be completed) : Grants from Dept of Defense and the Dept of Veterans Affairs

- **Abstract from the 2009 Congressionally Directed Medical Research Program meeting**

This study examined mechanisms of expression and extinction of fear conditioning, as an animal model of post-traumatic stress disorder, and also fear conditioning effects on drug reward. C57BL/6 mice were fear conditioned via repeated exposure to aversive stimuli (footshock co-terminating with a tone) or a sham procedure. After training, mice demonstrated freezing behaviors over 80% of the time when exposed to the context and tone cues (vs. 10%

freezing behavior in the control group). This was followed by fear extinction trials (20/day) via repeated exposure to context and cue without footshock; freezing behaviors returned to baseline levels. Brains from fear extinguished mice were harvested to measure synaptic plasticity using immunohistochemistry with the transcription factor FosB/delta FosB. FosB levels were elevated by more than a third in the prelimbic and infralimbic cortices (CX) of fear extinguished mice. No effects were found in the nucleus accumbens, anterior cingulate, basolateral amygdala, caudate-putamen. To measure effects on drug reward in fear conditioned mice, subjects were exposed to a standard conditioned place preference (CPP) paradigm using morphine (10 mg/kg SC) vs. saline conditioning. After CPP training, both sham and fear conditioned mice showed a preference for the morphine associated side. Fear extinction produces activation of neural plasticity factors in pre- and infralimbic CX and did not alter conditioned reward.